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# **Bariatric surgery is accompanied by changes in extracellular vesicle-associated and plasma Fatty Acid Binding Protein 4**

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**Keywords:** bariatric surgery, adipokines, lipolysis, weight loss, extracellular vesicles

**Short title:** Effects of bariatric surgery on EVs

**Manuscript type:** original contribution

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## **Abstract**

### **Background**

Bariatric surgery markedly reduces fat mass with beneficial effects on cardiometabolic health but the mechanisms involved are not fully understood. Extracellular vesicles (EVs) are secreted by a variety of cells, including adipocytes, and may mediate some of these benefits. However, the effects of bariatric surgery on circulating EVs are unclear.

### **Methods**

Concentration of plasma EVs isolated by ultracentrifugation at baseline, 1- and 6 months post-bariatric surgery (n=20) was established using Nanoparticle Tracking Analysis. EV origin (CD9: exosome; CD41: platelet; CD235a: erythrocyte; CD11b: leukocyte; CD144: endothelial), cytokine (interferon  $\gamma$ , interleukin-6, TNF $\alpha$ ) and adipocyte marker (adiponectin, FABP4, PPAR $\gamma$ ) expression was measured by Time Resolved Fluorescence immunoassay.

### **Results**

EV concentration and cell-of-origin markers (CD41, CD235a, CD11b, CD144) did not alter in response to surgery, neither did EV-expressed interferon $\gamma$ , IL-6, TNF $\alpha$ , adiponectin, PPAR $\gamma$  or CD9. EV-derived Fatty Acid Binding Protein 4 (FABP4) increased at 1 month (+49%) before returning to baseline by 6 months (-51%,  $p<0.05$ ), corresponding to similar changes in circulating plasma FABP4 (+22% and -24% at 1- and 6-months, respectively;  $p<0.001$ ). Patients who underwent biliopancreatic diversion had lower FABP4-expressing EVs at 6 months compared to those who underwent sleeve gastrectomy/gastric banding ( $p<0.05$ ), despite similar percentage weight reduction (-19% vs -20%, respectively). CD9 expression

correlated with EV-expressed FABP4, adiponectin, TNF $\alpha$  and Interferon  $\gamma$  ( $r=0.5$ ,  $r=0.59$ ,  $r=0.53$ ,  $r=0.41$  respectively,  $p<0.005$ ), suggesting transport by an EV population of exosomal rather than microvesicular origin.

## **Conclusions**

Bariatric surgery leads to a transient change in circulating EV- and plasma-derived FABP4, reflecting alterations in adipose tissue homeostasis.

## **Introduction**

The obesity pandemic has led to a greater adoption of bariatric surgery as a treatment for morbid obesity and its secondary complications [1]. Bariatric surgery exerts multiple effects on adipose tissue [2], including reduced adipocyte size, improved sensitivity to insulin, increased adiponectin and decreased levels of leptin, CRP, IL-6, TNF $\alpha$  and Monocyte Chemoattractant Protein-1 (MCP-1)[2]. However, the pathways regulating these changes are not fully understood.

Extracellular vesicles (EVs) are submicron particles secreted by most cell types during the cell cycle and particularly during cell stress [3,4]. EVs are released in one of two ways: intracellular formation of multivesicular exosomes which are subsequently released from

the plasma membrane, or as microvesicles (MVs) that are released directly from plasma membrane budding [3,4]. They contain cytoplasmic and membrane components of their 'donor' cell such as enzymes, proteins and microRNA [4] and are believed to affect recipient cell biology and gene transcription. EVs have also emerged as potential disease biomarkers [5]. Adipocyte-derived EVs (AD-EVs) are likely to play important roles in paracrine and endocrine communication within adipose tissue but comparatively little is known about them. They probably represent only a small percentage of the total population of EVs present in the circulation [6,7], at least in the healthy state, but are known to affect target cell activity [8,9], including increased lipid storage in small adipocytes[10].

Fatty Acid Binding Protein 4 (FABP4) is one of the most abundant cytoplasmic proteins within adipocytes, whose function extends beyond lipid transport to include regulation of glucose metabolism and inflammatory responses [6,11]. Recent data in 3T3-L1 adipocytes and mouse adipose tissue demonstrate that lipolytic stimuli trigger accumulation of FABP4 within intracellular multivesicular bodies (MVB), and its subsequent secretion via the non-classical pathway in vesicles expressing exosomal markers (CD63 and ALG-2 interacting protein X(ALIX))[7]. It appears, however, that only a small proportion of total FABP4 is transported in this way [6,7].

Since lipolysis triggers EV secretion from adipocytes, conditions associated with substantial weight loss might result in increased secretion of adipocyte-derived EVs into the circulation. We therefore hypothesised that bariatric surgery would result in increased expression of adipocyte-derived proteins, including FABP4, not only in a free form but also in association with EVs.

## Methods

### *Study populations*

EV characterisation was undertaken at 3 time-points in 20 patients undergoing elective bariatric surgery at The Welsh Institute of Metabolic and Obesity Surgery (WIMOS), Swansea, UK. Patients were aged 20-60 years and had a body mass index (BMI)  $>40\text{kg/m}^2$ . All subjects had type 2 diabetes (T2DM) or impaired glucose tolerance (IGT), diagnosed during an oral 75g glucose tolerance test (OGTT)[12]. Patients with any acute concurrent illness were excluded. Thirteen of the patients underwent restrictive laparoscopic surgery (sleeve gastrectomy (SG)  $n=10$ , gastric banding (GB)  $n=3$ ) and seven an open malabsorptive procedure (biliopancreatic diversion (BPD)).

### *Sample preparation*

Fasting venous blood was collected from a large peripheral vein into EDTA vacutainers. Samples were immediately spun twice at 3000g for 10mins to render plasma acellular, then frozen and stored at  $-80^{\circ}\text{C}$  until further analysis.

### *EV isolation and characterisation*

In order to isolate EVs, acellular plasma underwent 100,000g ultracentrifugation at  $4^{\circ}\text{C}$  for 1 hour. EVs were then re-suspended in 1x sterile phosphate-buffered saline (PBS) ( $0.22\mu\text{m}$  filtered). EV size and concentration were established by nanoparticle tracking analysis (NTA) using Nanosight LM10 (Malvern, UK), software version 3.1. For each sample, 60 second videos were recorded in replicates of 5. NTA determines the size and concentration of EVs

by analysing the Brownian motion and light scattering properties of the nanoparticle suspension illuminated by a laser beam [13].

Time Resolved Fluorescence (TRF) immunoassay [14,15] was used to establish EV cellular origin, cytokine and adipocyte-marker expression (CD41, CD11b, CD235a, CD144, CD9, IL-6, TNF $\alpha$ , interferon  $\gamma$ , adiponectin, FABP4; supplementary material).

#### *Other assays*

Fasting glucose was measured using a Roche Modular P800 Analyser. A Roche Modular E170 was used to measure fasting insulin. High sensitivity ELISA kits were used to evaluate IL-6 concentration (R&D Systems, Minneapolis, MN, USA). Plasma FABP4 concentration was established using a human FABP4 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) and plasma free fatty acids were measured by a Colorimetric Quantification Assay Kit (ab65341, Abcam, Cambridge, UK). All samples were assayed in duplicate.

#### *Statistical analysis*

This was a proof of concept study that had the primary aim of determining the change in plasma- and EV-expressed FABP4. There are very limited data available in relation to FABP4 in plasma following bariatric surgery and, to our knowledge, no studies investigating EV FABP4 in this context. As we anticipated a 20% change in plasma FABP4 over the three sampling points and a range of 10-15%, a power calculation suggested that we would need at least 15 subjects to detect this difference (one-way ANOVA at 3 levels;  $p=0.05$ ;  $\alpha=0.8$ ).



Normally distributed variables were reported as mean  $\pm$  SD whereas non-normally distributed variables were summarised as medians with interquartile ranges (IQR). For normally distributed variables the differences at different time points were analysed using repeated measured ANOVA with Bonferroni's multiple comparisons post-test analysis. For changes in variables with non-Gaussian distribution, the Kruskal-Wallis test was applied with post-test Dunn's comparison. Spearman's correlation analysis was applied to detect significant correlations between evaluated parameters. Statistical significance was accepted at  $p < 0.05$  and Graph Pad version 6.0 was used.

## **Results**

### *Population characteristics*

There were thirteen female and seven male patients, with a mean age of  $50.7 \pm 8$  years and a baseline mean body weight and BMI of  $151.3 \pm 31$  kg and  $54 \pm 12.6$  kg/m<sup>2</sup>, respectively. The anthropometric and metabolic characteristics are summarised in Table 1. As anticipated, all patients undergoing bariatric surgery achieved significant total weight loss (TWL) ( -8.3% at 1 month and -20% at 6 months to mean values of 138.8 kg and 121.6 kg, respectively;  $p < 0.001$ ) and BMI reduction of -10% and -18.5% (to mean values of  $48.9 \pm 11$  and  $44 \pm 10.2$  kg/m<sup>2</sup>, respectively;  $p < 0.001$ ).

### *EV concentration and content*

There were no significant differences in mean EV concentration/mL of plasma between baseline ( $1.44 \pm 0.9 \times 10^{11}$  nanoparticles/mL), 1 month ( $1.3 \pm 0.7 \times 10^{11}$ ) and 6 months postoperatively ( $1 \pm 0.4 \times 10^{11}$ ) (Figure 1a). However, detailed analysis of EV concentration within different size ranges (measured every 100nm) revealed a significant reduction in EVs measuring between 100 and 200nm in diameter at 6 months compared to baseline ( $p < 0.001$ ) (Figure 1b).

The relative distribution of the main EV cell-of-origin markers (CD41 (platelets), CD11b (monocytes/macrophages), CD235a (erythrocytes), CD144 (endothelial cells)) did not change in response to surgery (Figure 2).

Following surgery, there were no differences in median TRF signal for the exosomal marker CD9, EV-expressed adiponectin, PPAR $\gamma$ , IL6, TNF $\alpha$  or interferon  $\gamma$  (data not shown). However, the FABP4 EV signal increased (non-significantly) at 1 month before falling significantly by 6 months ( $p < 0.01$ ) (Figure 3a, 3b). The FABP4 EV signal at 6 months' follow-up was significantly lower in the group who underwent biliopancreatic diversion surgery ( $n=7$ ) compared to those who had sleeve gastrectomy or gastric banding ( $n=13$ ) ( $p < 0.05$ ) (Figure 3c), with no differences in EV FABP4 expression between those groups at baseline or 1 month.

A highly significant correlation was observed between the EV signal for CD9 and each of FABP4 ( $r=0.5$ ,  $p < 0.0005$ ), adiponectin ( $r=0.59$ ,  $p < 0.0001$ ), TNF- $\alpha$  ( $r=0.53$ ,  $p < 0.0001$ ) and interferon  $\gamma$  ( $r=0.41$ ,  $p < 0.005$ ) (Figures 3d-g), suggesting that these adipocytokines are transported by an EV population which is specifically of exosomal origin.

A similar pattern of change to that observed for EV FABP4 was observed with free plasma FABP4, with a significant rise at 1 month ( $p<0.001$  vs baseline) before a significant decrease by month 6 ( $p<0.001$  vs month 1) (Figure 4a, 4b). Furthermore, we observed that plasma FABP4 concentration correlated with plasma free fatty acid concentration at 1 month follow-up ( $r=0.48$ ,  $p<0.05$ ) but not at baseline or 6 months. Neither BMI nor plasma FABP4 correlated with EV FABP4 (Figure 4c, 4d).

In order to explore any relationship between EVs and weight loss, we subsequently subdivided our sample into two similar-sized groups: those who achieved more than 18% TWL at 6 months ( $n=9$ ) and those who did not ( $n=11$ ). Two significant differences were observed: significantly lower EV PPARY in the group that achieved greater weight loss at 6 months' follow-up, and significantly higher EV TNF $\alpha$  at 1 month follow-up in the group that failed to achieve weight loss of  $>18\%$ . In contrast, no relationships between any EV changes and improvements in glucose metabolism were observed (data not shown).

Since differences in fat distribution and lipolysis between men and women have been described [16], we also analysed our data split by gender. There were no gender differences in FFA concentrations at any time-point. Plasma FABP4 concentration at 6 months' follow-up was higher in females ( $78.47\pm14.9$  vs  $48.27\pm21$  ng/mL,  $p<0.05$ ) despite no significant EV FABP4 or BMI differences between genders at this time-point. EV CD11b (monocyte/macrophages marker) was higher in females at all time-points ( $p<0.05$ ). Baseline EV PPARY was also higher in females ( $p<0.05$ ); however, no differences were observed post-surgery. No other differences were observed in other EV markers.

## Discussion

Our study shows that bariatric surgery is followed by dynamic changes in the expression of EV-associated as well as free plasma FABP4. Since FABP4 is predominantly expressed in adipocytes, our finding of altered EV-expressed FABP4 after surgery is likely to reflect changes in adipocyte EV secretion. As demonstrated by Ertunc *et al*, lipolysis triggers a non-classical pathway of FABP4 secretion within EVs, and these EVs may be intended to play a different role in intercellular communication compared to their free circulating form [7]. Bariatric surgery is known to rapidly reduce visceral and subcutaneous lipid depots, accompanied by an increase in plasma free fatty acids and beta-hydroxybutyrate levels, reflecting increased lipolysis [17]. Previously, increased expression of lipolytic genes within adipose tissue following bariatric intervention was demonstrated, including triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and perilipin [18]. ATGL, HSL and free fatty acids have been found to affect FABP4 secretion [7].

In our study, EVs expressing FABP4 and plasma free FABP4 showed a similar pattern of change with a transient peak in the early postoperative period followed by significant reduction at 6 months' follow-up compared to 1 month. Furthermore, plasma FABP4 levels at 1 month correlated with plasma free fatty acid levels. We therefore believe that the transient postoperative rise in EV and plasma FABP4 may reflect the dynamic changes occurring in adipose tissue following surgery, including reduced total fat mass and adipocyte size, and increased lipolysis, albeit that a limitation of our work is that we did not measure plasma glycerol. Previous studies have shown similar changes in circulating FABP4 levels following weight loss [19, 20]. Stejskal *et al* indicated that this pattern of an initial rise in

plasma FABP4 with subsequent normalisation to baseline levels was observed particularly in individuals who achieved sustained weight loss [21]. However, we are unaware of any previous study evaluating EV-expressed and plasma free FABP4 simultaneously in the context of bariatric surgery.

Open bariatric surgery might involve more adipose tissue damage compared to a laparoscopic approach. However, at 1 month follow-up we did not observe differences in plasma and EV FABP4 in the BPD group compared to the SG/GB group. Nevertheless, it is entirely possible that differences in plasma- and EV-FABP4 between open and laparoscopic surgery might be apparent at very early time-points post-surgery.

We did not find a correlation between plasma FABP4 and the EV FABP4 TRF signal, nor between EV FABP4 and BMI. This might relate to the fact that adipocyte-derived FABP4-containing EVs probably carry only a small proportion of the total FABP4 secreted by adipocytes [6,7]. Moreover, adipocyte-derived EVs containing FABP4 could also be taken up by neighbouring cells in adipose tissue in a paracrine manner, as shown in previous studies [10]. Therefore, establishing a correlation between BMI and both plasma soluble FABP4 and EV-contained FABP4 is difficult. In contrast, EV-expressed TNF $\alpha$  was greatest at 1 month in subjects showing less weight loss. Whilst mechanisms other than weight loss may contribute to the changes in EV FABP4, it is tempting to speculate that EVs in patients showing the greatest weight loss exhibit a reduced inflammatory profile. However, this remains to be fully elucidated.

Previous studies examining the effects of bariatric surgery on circulating EVs have largely focused on changes in EV concentration and cell-of-origin expression, such as platelets,

monocytes or endothelial cells. Campello *et al* evaluated changes in a range of EV subtypes in 20 patients undergoing sleeve gastrectomy and found a significant decline in endothelial-, platelet-, and leukocyte-derived EVs and in annexin V, tissue factor and CD36-expressing EVs at 12 months' follow-up [22]. The percentage reduction in EV subtypes correlated with the reduction in BMI. Similarly, Cheng *et al* found a reduction in platelet-, endothelial-, monocyte- and tissue factor-expressing EVs in patients following Roux-en-Y gastric bypass surgery, with the monocyte EVs showing an association with HbA1c and BMI reductions [23]. In contrast, Stepanian *et al* found no effect of significant weight loss (mean BMI decrease of 24%, n=32, including 27 patients who had gastric bypass or sleeve gastrectomy) on total-, platelet- or endothelial-derived EVs, despite improvements in HOMA-IR, lipid profile and CRP [24]. This is in keeping with our findings, since we found no effect on platelet, leukocyte, erythrocyte or endothelial EVs nor overall EV concentration irrespective of the type of bariatric surgery performed and despite improvements in weight and glycaemic control. However, it should be noted that all of these referenced studies used flow cytometry to evaluate EV concentration and origin/protein expression. This approach is not sensitive enough to distinguish between different populations of EVs and to detect very small EVs (<200nm), as the analysis gate in standard flow cytometers is usually determined using 1µm beads. Therefore, the exosomal EV range which showed very interesting biological correlation in our data cannot be fully evaluated using flow cytometry alone. Identification of more precise changes pertaining to adipobiology that take place following the surgical approach are in line with the usefulness of new criteria to define success of bariatric and metabolic surgery that are being put forward.[25]

Similar to previous research evaluating EV changes post-bariatric surgery, the main limitation of our study includes the relatively small sample size. The study may therefore be underpowered to show significant differences in other EV cytokines. Secondly, many of our patients were taking concomitant medications at the time of study enrolment, including diabetes medication as, in contrast to most previous studies, we did not exclude patients with chronic illnesses such as hypertension or dyslipidaemia, which could have interfered with the EV readings. Thirdly, our study had a relatively short follow-up; studies with a longer follow-up period are needed in the future to fully describe the chronic changes in EVs post-bariatric surgery. Fourthly, it would have been helpful to have included a control group of patients undergoing non-bariatric surgery for comparison. Lastly, direct comparisons between clinical studies evaluating changes in circulating EVs are limited by the fact that establishing a gold standard method for EV isolation to be adopted across laboratories has only recently been a focus of the International Society of Extracellular Vesicles (ISEV) [26], although our adopted approach is one of the most commonly accepted protocols that we have introduced to yield consistent samples and results. This approach was not used in the studies by other authors [22-24] and similarly the EV analysis in the studies we cited was performed by flow cytometry which is typically not sensitive enough to detect EVs of exosomal origin and differs from our immunophenotyping method which we adapted for EV analysis specifically [14,15].

Despite the significant weight loss achieved by all study participants, the average 6 months' follow-up BMI remained within the class III obesity range (Table 1) which may partially explain why no significant differences in more of the EV markers were observed and why

the baseline and 6 months' follow-up plasma and EV-FABP4 results did not differ. Individuals who were selected to undergo BPD surgery had a higher average BMI at all 3 time-points compared to the SG/GB cohort, which corresponded with equally higher concentration of plasma FABP4 in this group ( $p<0.05$ ). Interestingly, however, the 6 months' follow-up EV FABP4 signal was significantly lower in the BPD arm compared to the SG/GB one, despite no differences at baseline or 1 month follow-up between these groups. Given that FABP4-expressing EVs are likely to derive from adipocytes, this may suggest that BPD has a greater effect on postoperative processes within adipose tissue regulating the secretion of this group of EVs. Although the mechanism behind this and the biological relevance of this phenomenon is not clear at present, this finding may be in keeping with previous reports indicating that this form of metabolic surgery might be more effective in reversing obesity-driven comorbidities such as type 2 diabetes, hypertension and dyslipidaemia[27,28]. Finally it should be noted that although increased circulating adipocyte-derived EVs suggest disturbed adipose tissue homeostasis, their full biological role has not been fully investigated and they may, depending on clinical circumstances, play a deleterious as well as protective role, as has been recently shown for circulating endothelial EVs in the context of vascular disease [29].

## **Conclusion**

To our knowledge, this study is the first to report concomitant fluctuations in plasma free FABP4 in addition to exosome-associated FABP4 secretion in the follow-up period after bariatric surgery which may relate, at least in part, to increased lipolysis. These changes are



likely to play an important role both in paracrine communication within adipose tissue and endocrine communication with other target tissues, since EVs are able to affect recipient cells' function and gene expression. Our observations suggest that adipocyte-derived EVs may be regarded as potential markers of adipose tissue health, and could represent a target for novel therapies aimed at improving metabolic health. However, further research is required in order to understand the precise mechanisms regulating secretion and content of adipocyte-derived EVs.

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**Conflict of interests:** The authors declare that they have no conflict of interest

**Ethical Approval:** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Local Research Ethics Committee (LREC No: 06/WMW02/7).

**Informed consent:** Informed consent was obtained from all individual participants included in the study.

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#### **Figure legends:**

**Figure 1:** a) EV concentration/mL of plasma at baseline, 1 month and 6 month follow-up (mean±SEM) b) 2-way ANOVA with Bonferroni post-test revealed significant decrease in EVs sized 100.5-199.5nm in diameter at 6 months' follow-up compared to baseline (mean±SD),  $p<0.01$  (\*\*)

**Figure 2:** Bariatric surgery did not alter EV cellular origin. Relative expression at baseline, 1 and 6 months' postoperatively: CD41 (platelets):  $25\pm13\%$  vs  $27\pm20\%$  and  $27\pm17\%$ ; CD11b

(macrophages/monocytes):  $51 \pm 25\%$  vs  $51 \pm 26\%$  and  $54 \pm 24\%$ ; CD235a (erythrocytes):  $10 \pm 6\%$  vs  $11 \pm 10\%$  and  $9 \pm 5\%$ ; CD144 (endothelial cells):  $15 \pm 10\%$  vs  $16 \pm 12\%$  and  $15 \pm 13\%$ , respectively, all  $p$ =non-significant.

**Figure 3:** a) Changes in EV FABP4 (median with IQR) reveal significantly lower TRF signal at 6 months' follow-up compared to 1 month ( $p < 0.05$ ) b) Individual pattern of changes in EV FABP4 TRF signal for all study participants at 3 time points c) Median EV FABP4 signal was significantly lower in the cohort that underwent biliopancreatic bypass diversion surgery at 6 month follow-up ( $p < 0.05$ ). Spearman's correlation analysis revealed significant correlation between the exosomal marker CD9 and EV-derived: FABP4 (d), adiponectin (e), TNF $\alpha$  (f) and interferon  $\gamma$  (g).  $*$ = $p < 0.05$ ,  $**$ =  $p < 0.01$

**Figure 4:** a) Plasma free FABP4 levels (means) were significantly higher at 1 month follow-up compared to baseline and 6 months' follow-up b) Plasma FABP4 concentration at 3 time points for all study participants ( $n=20$ ) c) Spearman's correlation analysis revealed significant correlation between plasma FABP4 and BMI ( $r=0.6$ ,  $p < 0.0001$ ) d) No correlation between EV-contained and plasma free FABP4 was observed ( $r=0.05$ ,  $p$ =ns) e) Significant correlation was observed between plasma FABP4 and plasma free fatty acids at 1 month follow up ( $r=0.48$ ,  $p < 0.05$ ).  $*$ = $p < 0.05$ ,  $**$ =  $p < 0.01$ ,  $***$ = $p < 0.001$

## **Supplementary material- methodology**

### *Time resolved fluorescence immunoassay*

High affinity protein binding 96 wells plates were loaded with  $5 \times 10^9$  EVs/well diluted to a total volume of 100  $\mu$ L with x1 filtered PBS and incubated overnight at 4°C. Following a wash with Delfia Buffer (Perkin Elmer, Waltham, MA, USA), non-specific sites were blocked with 1% (wt/vol) Bovine Serum Albumin (BSA) in PBS for 2 hours. Following a further wash, primary antibodies were added in duplicates at 3  $\mu$ g/mL concentration (anti-CD41, anti-CD11b, anti-CD235a, anti-CD144, anti-CD9, anti-IL-6, anti-TNF $\alpha$ , anti-interferon  $\gamma$ , anti-adiponectin, anti-FABP4 and anti-PPAR $\gamma$ ). After overnight incubation, secondary antibody (anti-rabbit IgG Biotin labelled, 1:2500 in 0.1 % (wt/vol) BSA in PBS) was added, followed by staining with Europium-labelled Streptavidin (Perkin Elmer, Waltham, MA, USA). Delfia Enhancement Solution (Perkin Elmer, Waltham, MA, USA) was then added leading to formation of fluorescent chelates. The plate was measured using a CLARIOstar spectrometer (BMG Labtech Ltd, Aylesbury, UK) configured to Time Resolved Fluorescence (TRF).

All primary rabbit monoclonal antibodies were supplied by Abcam (Cambridge, UK) apart from anti-CD9 antibody which was provided by Cell Signalling Technology (Danvers, AM, USA). For the analysis of internal EV content, RIPA Lysis Buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) containing protease inhibitor, sodium orthovanadate and phenylmethylsulfonyl fluoride (PMSF) was added for 1 hour before the addition of primary antibodies to enable the disruption of the EV membrane.